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Hypoxyprobe™ PAb Plus Kit

(HPI Part # HP9-XXX)

Kit contents:

Solid pimonidazole HCl (Hypoxyprobe™-1)

**FITC conjugated to anti-pimonidazole rabbit polyclonal antibody (FITC-PAb) and
Rabbit anti-FITC anti dye conjugated with horseradish peroxidase as a secondary reagent.**

Applications: Immunochemical detection of cell and tissue hypoxia including immunofluorescence, immuno-peroxidases or flow cytometry. There is very low background signal from naturally green fluorescing proteins (that would usually interfere with the hypoxic signal) in rat tissues when the Hypoxyprobe™-1 Plus Kit is combined with an anti-FITC secondary reagent.

Quantities: a. The 3 different size Hypoxyprobe™-1 Plus Kits contain either 100 mg, 200 mg or 1000 mg of pimonidazole HCl. A dosage of 60mg/kg body weight is sufficient for animal studies.

b. The 3 different size Hypoxyprobe™-1 Plus Kits also contain One vial (100 and 200 mg Kits) or two vials (1000 mg Kit) containing 200 uL of an anti-pimonidazole, FITC-conjugated rabbit polyclonal antibody (Lot# R2710-B). Each vial contains 200 microliters of a 0.50 mg/ml solution of the FITC-PAb in PBS containing 1 % BSA and 0.09% sodium azide. Optimal dilution of the FITC-PAb is to be determined by the investigator but a 1:50-100 dilution has been found to give strong immunostaining in rat tumor tissue when combined with a 1:50-100 dilution of the peroxidase conjugated anti-FITC secondary reagent. While not designed specifically for immunofluorescence,

FITC-PAb can be used for this purpose. Antibody dilution is typically lower for immunofluorescence than for immunoperoxidase detection.

Not supplied: Standard reagents used for immunohistochemical analyses.

Storage:

- Store Hypoxyprobe™-1 solid at room temperature in the dark (can also be stored at 2-8 degrees C).
- Store FITC-PAb and HRP conjugated rabbit anti-FITC at 2-8 degrees C in the dark.

Do not freeze.

Detailed Description of Hypoxyprobe™-1 Plus Kit components

1) Hypoxyprobe™-1 is a substituted 2-nitroimidazole whose chemical name is pimonidazole hydrochloride with a molecular weight of 290.8; a water solubility of 400 millimolar (116 mg/ml); and, ultraviolet absorbance at 324 nm (extinction coefficient 7020 in 0.9% saline). The free base, pimonidazole, has a molecular weight of 254.3, a pKa of 8.7 and an octanol water partition coefficient of 8.5. See www.hypoxyprobe.com for mechanism of action, frequently asked questions (FAQ) and applications for Hypoxyprobe™-1 kits.

Solid Hypoxyprobe™-1 has been stored for two years at room temperature in subdued light and stored in 0.9% saline (100 gms/liter) at 4°C for 4.5 years without detectable degradation (UV and HPLC analyses).

Pimonidazole is reductively activated in hypoxic cells and forms stable adducts with thiol (sulphydryl) groups in proteins, peptides and amino acids. FITC-PAb binds to these adducts allowing their detection by immunochemical means.

2) FITC-PAb, a fluorescein-conjugated, anti-pimonidazole, rabbit polyclonal antibody (Lot# R2710-B), is supplied at a concentration of 0.5 mg/ml in PBS containing 0.09% proclin as a stabilizer. Tissues of interest can be studied by immunohistochemistry on frozen fixed sections or formalin fixed paraffin embedded sections or by flow cytometry following tissue disaggregation.

Note: FITC-PAb binds to protein, peptide and amino acid adducts of pimonidazole but tissue processing during immunohistochemistry washes away peptide and amino acid adducts so that immunohistochemical hypoxia detection relies on protein adducts of pimonidazole in hypoxic tissue.

3) The chromogenic anti-FITC secondary reagent is an affinity-purified rabbit IgG polyclonal anti dye protein conjugated to horseradish peroxidase that has been prepared for Hypoxyprobe, Inc. The peroxidase conjugated anti-FITC secondary protocol provides strong immunostaining with very low background signal from any naturally green fluorescing proteins present in formalin fixed, paraffin embedded rat tumor tissue.

Assay Instructions

1. Investigations of normal or tumor tissue hypoxia begin with the intravenous infusion, intraperitoneal injection or oral ingestion of a HypoxyprobeTM-1 (pimonidazole HCl) solution at a dosage of 60 mg/kg body weight. For a 25g rat this amounts to 1.5 mg/rat. Dosages up to 400 mg/kg have been used in mice without detectable toxicity or change in tissue hypoxia but 60 mg/kg gives good immunostaining at minimum cost.

The solubility of HypoxyprobeTM-1 in saline is 116 mg/ml so that very small volumes can be used to administer HypoxyprobeTM-1. Following injection or ingestion, pimonidazole is distributed to all tissues including brain but it forms adducts with thiol containing proteins only in those cells that have a oxygen concentration less than 14 micromolar -- equivalent to a partial pressure $pO_2 = 10$ mm Hg at 37°C. In addition to tumors, normal tissues such as liver, kidney and skin possess cells at, or below, a $pO_2 = 10$ mmHg. These normal tissues bind HypoxyprobeTM-1.

The plasma half-life of HypoxyprobeTM-1 in mice is approximately 25 minutes (see the FAQ link at www.hypoxyprobe.com for references). For comparison, plasma half-lives for rats is 45 minutes, dogs 90 minutes and humans 300 minutes. Rat tissues of interest may be harvested 15 to 90 minutes after HypoxyprobeTM-1 administration. HypoxyprobeTM-1 residing in tissues at the time of harvest will be bound when dissected tissues go anoxic but the amount of residual HypoxyprobeTM-1 is very small compared to the amount that tissues are exposed to during a 15 to 90 minute experiment so that any non-specific binding due to residual HypoxyprobeTM-1 is undetectable.

In addition to animal studies, HypoxyprobeTM kits can be used for cells in tissue culture - See Applications link at [Knowledge Center \(hypoxyprobe.com\)](http://Knowledge Center (hypoxyprobe.com)). Typically, cell suspensions are incubated under hypoxia for 1 to 2 hours in the presence of 100 to 200 micromolar HypoxyprobeTM-1. The cells are harvested by cytopsin, fixed and immunostained with FITC-PAb and the anti-FITC chromogenic secondary reagent.

Figure. Immunoperoxidase staining for pimonidazole binding in a formalin-fixed paraffin embedded tissue section from a rodent tumor using 1:100 dilution of FITC-PAb and 1:100 dilution of horseradish peroxidase conjugated rabbit anti-FITC IgG.

Suggested protocol for immunostaining pimonidazole adducts in formalin-fixed, paraffin-embedded tissue sections using FITC-conjugated anti-pimonidazole rabbit polyclonal antibody and peroxidase-conjugated anti-FITC rabbit secondary antibody.

Step	Procedure	Time, Min	Temp	Reagent	Note
1	Soften paraffin	20	40°C	None	
2	Dewax, Dip and Blot x10	2	RT	Clear Rite 3	1
3	Rehydrate, Dip and Blot x10	2	RT	100% Ethanol	
4	"	2	RT	95% Aqueous ethanol	
5	"	2	RT	80% Aqueous ethanol	
6	"	2	RT	0.2% Brij 35 in distilled water	2
7	"	2	RT	PBS+ 0.2% Brij 35	3
8	Quench tissue peroxidase	5	RT	3% H ₂ O ₂ in distilled water	4
9	Wash with rinse buffer	2	RT	PBS+ 0.2% Brij 35	
10	Antigen Retrieval	20	90°C	Target retrieval reagent	5
11	Cool to RT	20	RT	None	
12	Wash with rinse buffer	2	RT	PBS + 0.2% Brij 35	
13	Block non-specific binding	10	RT	Protein blocking agent	6
14	Apply primary MAb	60	RT	FITC-PAb (1:50-100)	7
15	Wash with rinse buffer	2	RT	PBS+ 0.2% Brij 35	
16	Apply anti-FITC secondary	30	RT	HRP linked to rabbit anti-FITC (1:50-100)	8
17	Wash with rinse buffer	2	RT	PBS+ 0.2% Brij 35	
18	Peroxidase chromogen	10	RT	DAB	9
19	Stop DAB reaction	1	RT	Distilled water flush	
20	Counterstain	0.5	RT	Hematoxylin	10
21	Cover tissue section	45	45°C	CC/Mount	11

Technical Notes

1. Clear-Rite 3 -- a less toxic alternative to xylene -- is available from VWR (Cat# 84000-052). For dewaxing (step 2) and tissue rehydration (steps 3-7), ProbeOn Plus slides (Fisher Scientific; Cat# 15-188-52) are held vertically in a MicroProbe Staining Station (Fisher Scientific) in pairs with tissue sections facing each other. The paired slides are dipped in solvent

allowing capillary action to carry solvents over the tissue sections. Solvent is removed by blotting the lower end of the slide pair on adsorbent filter paper. The dip and blot procedure is repeated a total of 10 times for each of steps 2-7.

Note: These steps were designed for the MicroProbe Staining Station but other routine IHC procedures can be used.

2. Brij 35 is enzyme grade polyoxyethylene(23)lauryl ether available from Fisher Scientific (Cat# BP345-500). Alternatives to Brij35 such as Tris buffered saline (TBS) available from Chemicon International, Temecula, CA (Cat# 20845) can be used as the rinse solution. It is recommended that Tween 20® be added to the TBS rinse buffer at a final concentration of 0.1%.
3. PBS is 10 mM phosphate buffered saline prepared from tablets available from Sigma (Cat# P-4417), for example.
4. 3% H₂O₂ is diluted Analytical Reagent grade 31.3% H₂O₂ available from Malinckrodt Baker, Paris, KT (Cat# 5240). Commercial peroxidase blocking agents can be used.
5. Antigen retrieval agents such as BIORAD Cat# BUF025B; Chemicon International Cat# 21545; or, DAKO Cat# S2369; can be used. Pimonidazole protein adducts are very robust so that the antigen retrieval reagent can be chosen on the basis of requirements for other factors of interest in tissue sections.
6. Any serum free protein blocker such as that from DAKO Corp. (Carpinteria, CA)(Cat# X0909) can be used. Note that the protein block is not washed from the section but flicked off to leave residual protein block on the section.
7. The primary reagent is a FITC conjugated, anti-pimonidazole rabbit polyclonal antibody (FITC-PAb). The concentration of the FITC-conjugated rabbit polyclonal antibody is 0.5 mg/ml and the FITC to protein molecular ratio is typically 4:1. The investigator will determine the optimum dilution but a 1:50-100 dilution in antibody diluent (e.g., Chemicon International, Cat# 21544) gives strong immunostaining with low background for formalin fixed, paraffin embedded, tissue sections. Typically, 100 microliters of diluted FITC-PAb is applied to each tissue section. FITC-PAb can be used on tissue sections from all species including mice.
8. A rabbit, horseradish peroxidase-conjugated, secondary reagent is included in the Hypoxyprobe Plus Kit but any suitably labeled, secondary anti-FITC antibody can be used. A 1:50-100 dilution of the secondary rabbit anti-FITC horseradish peroxidase conjugated antibody in the Hypoxyprobe-1 Plus Kit is typically used.
9. Any commercially available liquid 3,3'-diaminobenzidine reagent (DAB) is suitable including DAB A and B reagents from Chemicon International (Cat# 71895 and 71896, respectively).
10. Any commercially available hematoxylin counterstaining reagent is suitable including Chemicon International Cat# 20844.
11. CC/Mount (Sigma; Cat# C9368), a direct replacement for Biomed's Crystal Mount, is an aqueous based permanent mount for immunostained sections. Alternatives include cover slipping with PermOUNT (Fisher Scientific; Cat# SP15-500).